

Fluctuation analysis with cell deaths

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SUMMARY

The classical Luria-Delbrück model for fluctuation analysis is extended to the case where cells can either divide or die at the end of their generation time. This leads to a family of probability distributions generalizing the Luria-Delbrück family, and depending on 3 parameters: the expected number of mutations, the relative fitness of normal cells compared to mutants, and the death probability of mutants. The probabilistic treatment is similar to that of the classical case; simulation and computing algorithms are given. The estimation problem is discussed: if the death probability is known, the two other parameters can be reliably estimated. If the death probability is unknown, the model can be identified only for large samples.

Key words: Bellman-Harris branching process; cell kinetics; fluctuation analysis; Luria-Delbrück distribution; mutation model

1. INTRODUCTION

Since it appeared more than 60 years ago, the Luria-Delbrück distribution has been widely used as a model for the occurrence of mutants in cell cultures: see chap. II p. 59 of [Kendall \(1952\)](#) for an early review, and [Zheng, 1999, 2010](#) for more recent ones. It is obtained as a limit when the initial number of cells and the experiment time are large, and the mutation probability is small. One of the underlying hypotheses is that cells only divide and never die, which is untrue in reality, even though the probability of death has been estimated to rather low values ([Stewart and others, 2005](#); [Fontaine and others, 2008](#)). A Markovian model of mutations including cell deaths was considered by [Tan \(1982\)](#), who proposed a computing algorithm for the distribution of mutants. [Angerer \(2001, section 3\)](#) also discussed the influence of cell death on the distribution of mutants. To the best of our knowledge no explicit representation of the distribution of mutants in a general model including cell deaths, and no quantitative study of the influence of deaths on the estimation of parameters have appeared so far. Our objective here is to extend the classical Luria-Delbrück model to the case where cells have a certain probability to die rather than divide, and provide statistical tools for the estimation of the parameters.

Our hypotheses are the following:

- at time 0 a homogeneous population of n normal cells is given;
- the generation time of any normal cell is a random variable with distribution G ;
- upon completion of the generation time of a normal cell:
 - with probability p one normal and one mutant cell are produced;
 - with probability q the cell dies out;
 - with probability $1 - p - q$ two normal cells are produced,
- the generation time of any mutant cell is exponentially distributed with parameter μ^* ;

- upon completion of the generation time of a mutant cell:
 - with probability δ the cell dies out;
 - with probability $1 - \delta$ two mutant cells are produced,
- all random variables and events (division times, mutations, and deaths) are mutually independent.

Consider an initial (large) number n of normal cells. Assume that the mutation probability p is small, that the time t at which mutants are counted is large, and that the asymptotics are such that the expected number of mutations α before time t is non null and finite (precise hypotheses and statements will be given in section 2). Denote by ν and μ the exponential growth rates of normal and mutant cells respectively, and by $\rho = \nu/\mu$ the *relative fitness*. It will be shown that the total number of mutants at time t approximately follows an integer valued distribution, whose probability generating function (PGF) is given by:

$$g_{\alpha, \rho, \delta}(z) = \exp(\alpha(h_{\rho, \delta}(z) - 1)) , \quad (1.1)$$

where:

$$h_{\rho, \delta}(z) = \int_0^1 \frac{\delta(1-z) + v((1-\delta)z - \delta)}{(1-\delta)(1-z) + v((1-\delta)z - \delta)} \rho v^{\rho-1} dv . \quad (1.2)$$

The parameters are:

1. α : the expected number of mutations
2. ρ : the relative fitness of normal cells compared to mutants.
3. δ : the death probability of a mutant cell.

For $\delta = 0$, the Luria-Delbrück distribution with parameters α and ρ , or $\text{LD}(\alpha, \rho)$, is obtained as a particular case. We propose to name “Luria-Delbrück with deaths”, and denote by $\text{LDD}(\alpha, \rho, \delta)$,

the distribution on integers with PGF $g_{\alpha,\rho,\delta}$. We propose a statistical study of the $\text{LDD}(\alpha, \rho, \delta)$ including:

- fast simulation algorithm,
- computation of probabilities,
- asymptotic probabilities for large values,
- point estimation of parameters,
- confidence intervals.

We have developed in R ([R Development Core Team \(2008\)](#)) a set of functions that perform the usual operations on the LDD distributions (simulation, distribution function and quantile computation), output estimates and confidence intervals. These functions have been made available online: <http://www.ljk.imag.fr/membres/Bernard.Ycart/LD/>.

The paper is organized as follows. In section 2, the theoretical justification of the model is given. It is based on standard results from branching process theory. A simple probabilistic interpretation will be given. Section 3 describes the simulation and computation algorithms of the $\text{LDD}(\alpha, \rho, \delta)$: they are quite similar to those known for the $\text{LD}(\alpha, \rho)$ [Zheng \(2005\)](#). The estimation problem is addressed in section 4. The proposed method is based on generating function estimates, extending those proposed for the $\text{LD}(\alpha, \rho)$ in [Hamon and Ycart \(2012\)](#). Experimental results, both on simulated and real data are reported in section 5.

2. ASYMPTOTICS FOR NUMBERS OF MUTANTS

The results exposed in this section are applications of the general theory of supercritical age-dependent continuous time branching processes (or Bellman-Harris processes): see [Harris \(1963, Chap. VI\)](#) and [Athreya and Ney \(1972, Chap. IV\)](#) as general references. They are similar to those

detailed in section 2 of [Hamon and Ycart \(2012\)](#), and we shall mainly develop those differences with the classical model, that arise from taking cell deaths into account.

Firstly, consider the number of normal cells as a function of time. Recall that the generation times are assumed to be independent and identically distributed (i.i.d.) random variables, with common distribution G . Upon completion of a generation time, the number of (normal) offspring is:

- 0 with probability q (death),
- 1 with probability p (mutation),
- 2 with probability $1 - p - q$ (division).

Therefore the PGF of the offspring distribution is:

$$q + pz + (1 - p - q)z^2 ,$$

and its expectation (mean offspring number) is $m = p + 2(1 - p - q)$. We shall assume that the mean offspring number is larger than 1, so the corresponding clones may survive with positive probability (supercritical case). If one normal cell is initially present, then either the population dies out or it grows exponentially. The probability that it dies out is the smallest positive root of the equation $z = q + pz + (1 - p - q)z^2$, which we shall denote by ε :

$$\varepsilon = \frac{q}{1 - p - q} .$$

With probability $1 - \varepsilon$ the clone does not die out, in which case it grows exponentially. The exponential growth rate (or *Malthusian parameter*) ν is defined as the unique root of the equation:

$$m \int_0^{+\infty} e^{-\nu s} dG(s) = 1 . \tag{2.3}$$

Theorem 17.1 p. 142 of [Harris \(1963\)](#) gives a precise meaning to the expression “exponential growth”. It states that:

$$\lim_{t \rightarrow +\infty} \mathbb{E}[N_t \mid N_0 = 1, N_t > 0] e^{-\nu t} = C ,$$

where N_t denotes the number of normal cells at time t . The limit is the proportionality coefficient of exponential growth. It is given by:

$$C = \left(\nu \frac{m^2}{m-1} \int_0^{+\infty} s e^{-\nu s} dG(s) \right)^{-1}. \quad (2.4)$$

Assume n normal cells are present at time $t = 0$. Let (t_n) be a sequence of instants, tending to infinity as n tends to infinity. At large time t_n , a proportion ε of the clones stemming from the n initial cells will have died out. A proportion $1 - \varepsilon$ grow exponentially with rate ν . So the final number of normal cells will be asymptotically equivalent to $n(1 - \varepsilon)C e^{\nu t_n}$.

Consider now mutations. Let (p_n) be a sequence of mutation probabilities, tending to 0 as n tends to infinity. Since (p_n) tends to zero, mutations have an asymptotically null effect on the growth rate of the population. Indeed the mean offspring number tends to $2(1 - q)$, the growth rate tends to the unique solution ν of the equation:

$$2(1 - q) \int_0^{+\infty} e^{-\nu s} dG(s) = 1,$$

and the proportionality constant tends to:

$$C = \left(\nu \frac{4(1 - q)^2}{1 - 2q} \int_0^{+\infty} s e^{-\nu s} dG(s) \right)^{-1}.$$

Moreover, since the number of divisions occurring in dying clones remains bounded, they can be neglected, and it can be considered that mutants observed at time t_n only come from divisions in surviving mutant clones. Their number is asymptotically equal to the final number of normal cells, i.e. $n(1 - \varepsilon)C e^{\nu t_n}$. Assume now that:

$$\lim_{n \rightarrow +\infty} p_n n(1 - \varepsilon)C e^{\nu t_n} = \alpha, \quad (2.5)$$

where α is some fixed positive real. The expected number of mutations tends to α , and since mutations are supposed to occur randomly, their number asymptotically follows the Poisson distribution with parameter α , by the law of small numbers. Notice that the interpretation of α

as the product of the mutation probability by the final number of cells holds whether cell deaths are considered or not; thus estimating α permits to estimate the mutation probability p , dividing the estimate of α by the final number of cells, exactly as in classical fluctuation analysis.

Consider now the durations between random split times of non dying clones, and the final time t_n : we call them *split lags*. Theorem 2.1 p. 669 of [Kuczek \(1982\)](#) states the almost sure convergence of the empirical distribution of split lags, to the distribution function of the exponential with parameter ν . From section 3 of that same article, it follows that the developing times of a fixed number k of mutant clones converge in distribution to the product of k independent copies of the exponential distribution with parameter ν .

Let us now turn to mutant clones, i.e. populations of mutant cells stemming from a single initial mutant cell. Recall that the generation times of mutants are assumed to be exponentially distributed with rate μ^* . The number of mutants at time s is a linear growth birth-and-death process. The rates are proportional to the number k of cells in the population, the death rate being $\mu^*\delta k$ and the birth rate being $\mu^*(1 - \delta)k$. We shall assume also that mutant clones may survive with positive propability, which occurs only if $\delta < 1/2$. The exponential growth rate of mutant clones is the difference between birth and death individual rates:

$$\mu = \mu^*(1 - \delta) - \mu^*\delta = \mu^*(1 - 2\delta) .$$

The distribution at time s of the number of mutant cells, stemming from a single mutant cell at time 0 is explicitly known, and characterized by the following PGF: [Athreya and Ney \(1972, p. 109\)](#).

$$F(z, s) = \frac{\delta(1 - z) + e^{-\mu t}((1 - \delta)z - \delta)}{(1 - \delta)(1 - z) + e^{-\mu t}((1 - \delta)z - \delta)} . \quad (2.6)$$

Let us summarize the 3 main arguments:

- A1: the number of mutations converges in distribution to the Poisson distribution with parameter α ;

A2: the joint distribution of the developing times of a fixed number k of mutant clones converges in distribution to the product of k independent copies of the exponential distribution with parameter ν ;

A3: the size at time s of a mutant clone has distribution with PGF $F(z, s)$.

From A2, the size of any mutant clone is an exponential mixture, the PGF of which can be expressed using (2.6) as:

$$\int_0^{+\infty} \frac{\delta(1-z) + e^{-\mu t}((1-\delta)z - \delta)}{(1-\delta)(1-z) + e^{-\mu t}((1-\delta)z - \delta)} \nu e^{-\nu s} ds .$$

Changing $e^{-\mu s}$ into v yields the expression (1.2) of $h_{\rho, \delta}$. The distribution with PGF $h_{\rho, \delta}$ can be seen as a two-parameter extension of the Yule distribution with parameter ρ : it will be denoted by $YD(\rho, \delta)$ (for “Yule with deaths”). From A1, the total number of mutants is the sum of a random number of sizes of independent random clones, each with $YD(\rho, \delta)$ distribution: the resulting distribution is a compound Poisson with parameter α and base $YD(\rho, \delta)$, hence the expression (1.1) of the PGF $g_{\alpha, \rho, \delta}$ of the $LDD(\alpha, \rho, \delta)$.

3. PROBABILITY CALCULATIONS

Computation and simulation algorithms for the $YD(\rho, \delta)$ and the $LDD(\alpha, \rho, \delta)$ distributions are described in this section. The probabilities of the $YD(\rho, \delta)$ and $LDD(\alpha, \rho, \delta)$ will be denoted by $(p_k)_{k \in \mathbb{N}}$ and $(q_k)_{k \in \mathbb{N}}$ respectively.

$$h_{\rho, \delta}(z) = \sum_{k=0}^{+\infty} p_k z^k \quad \text{and} \quad g_{\alpha, \rho, \delta}(z) = \sum_{k=0}^{+\infty} q_k z^k .$$

We begin with a probabilistic interpretation of the distribution at time s of mutant clones, the PGF $F(z, s)$ of which is given by (2.6). Let us rewrite $F(z, s)$ as:

$$F(z, s) = \frac{\delta(1 - e^{-\mu s}) - z(\delta - e^{-\mu s}(1 - \delta))}{(1 - \delta - \delta e^{-\mu s}) - z((1 - \delta)(1 - e^{-\mu s}))} .$$

An easy series expansion yields the corresponding probabilities:

$$F(z, s) = \sum_{k=0}^{+\infty} p_k(s) z^k ,$$

with

$$p_0(s) = \frac{\delta(1 - e^{-\mu s})}{1 - \delta - \delta e^{-\mu s}} ,$$

and for $k \geq 1$,

$$p_k(s) = (1 - p_0(s))\pi(s)(1 - \pi(s))^{k-1}, \text{ with } \pi(s) = \frac{(1 - 2\delta)e^{-\mu s}}{1 - \delta - \delta e^{-\mu s}} . \quad (3.7)$$

In other words, a random variable with PGF $F(z, s)$ is a random mixture: either 0 with probability $p_0(s)$ or (with probability $1 - p_0(s)$), a geometric random variable with parameter $\pi(s)$. The $YD(\rho, \delta)$ is an exponential mixture of these distributions. Using again the change of variable $e^{-\mu s} \mapsto v$,

$$p_0 = \int_0^1 \frac{\delta(1 - v)}{1 - \delta - \delta v} \rho v^{\rho-1} dv , \quad (3.8)$$

and for $k \geq 1$:

$$p_k = (1 - \delta)^{k-1} (1 - 2\delta)^2 \int_0^1 \frac{(1 - v)^{k-1}}{(1 - \delta - \delta v)^{k+1}} \rho v^\rho dv . \quad (3.9)$$

The integral in (3.9) can be computed numerically up to rather large values of k . An equivalent for larger k 's can be calculated as follows. Rewrite (3.9) as:

$$\begin{aligned} p_k &= \left(\frac{1 - 2\delta}{1 - \delta} \right)^2 \int_0^1 \frac{(1 - v)^{k-1}}{(1 - v \frac{\delta}{1 - \delta})^{k+1}} \rho v^\rho dv \\ &= k^{-\rho-1} \left(\frac{1 - 2\delta}{1 - \delta} \right)^2 \int_0^k \frac{(1 - \frac{u}{k})^{k-1}}{(1 - \frac{u}{k} \frac{\delta}{1 - \delta})^{k+1}} \rho u^\rho du \end{aligned}$$

The following equivalent is obtained.

$$p_k \underset{k \rightarrow \infty}{\sim} k^{-\rho-1} \left(\frac{1 - 2\delta}{1 - \delta} \right)^{1-\rho} \rho \Gamma(\rho + 1) . \quad (3.10)$$

A well known algorithm expresses the q_k 's as a function of the p_k 's ([Embrechts and Hawkes, 1982](#); [Pakes, 1993](#)).

$$q_0 = e^{-\alpha(1-p_0)}, \quad \text{and for } k \geq 1, \quad q_k = \frac{\alpha}{k} \sum_{i=1}^k i p_i q_{k-i} . \quad (3.11)$$

The proof of (3.11) is easy:

$$\begin{aligned}
\frac{dg_{\alpha,\rho,\delta}}{dz} &= \alpha \frac{dh_{\rho,\delta}}{dz} g_{\alpha,\rho,\delta} \\
&= \alpha \left(\sum_{i=1}^{+\infty} i p_i z^{i-1} \right) \left(\sum_{k=0}^{+\infty} q_k z^k \right) \\
&= \sum_{k=1}^{+\infty} k q_k z^{k-1} .
\end{aligned}$$

The equivalent of q_k is deduced from subexponential theory: [Embrechts and Hawkes \(1982, Theorem 1\)](#).

$$q_k \underset{k \rightarrow \infty}{\sim} \alpha p_k \underset{k \rightarrow \infty}{\sim} \alpha k^{-\rho-1} \left(\frac{1-2\delta}{1-\delta} \right)^2 \rho \Gamma(\rho+1) . \quad (3.12)$$

For any δ , a heavy tail distribution with tail exponent ρ is obtained.

Another consequence of the probabilistic interpretation is a simulation algorithm for the YD(ρ, δ) and the LDD(α, ρ, δ).

For the YD(ρ, δ):

- simulate a random time s , according to the exponential distribution with parameter ρ ;
- compute $p_0(s)$ and $\pi(s)$;
- make a random choice:
 - with probability $p_0(s)$, output 0,
 - with probability $1 - p_0(s)$, output a geometric random number with parameter $\pi(s)$.

For the LDD(α, ρ, δ):

- simulate a random integer n according to the Poisson distribution with parameter α ;
- simulate a sample of size n of the YD(ρ, δ),

- output the sum of that sample.

These two algorithms have been encoded in R, and the simulation functions are included in the script available online.

4. PARAMETER ESTIMATION

This section addresses the problem of parameter estimation. The main difficulty comes from the fact that two LDD distributions may be quite close for rather different sets of parameters; this makes the model hardly identifiable in practice. In order to evaluate the actual identifiability, we proceed as follows. Let α_0 and ρ_0 be two given positive values, and let (q_0, q_1) be the first two probabilities of the $\text{LDD}(\alpha_0, \rho_0, 0)$. For any value $\delta < 0.5$, there exists a couple $(\alpha_\delta, \rho_\delta)$ such that the first two probabilities of the $\text{LDD}(\alpha_\delta, \rho_\delta, \delta)$ coincide with (q_0, q_1) . It turns out that the whole distribution $\text{LDD}(\alpha_\delta, \rho_\delta, \delta)$ is very close to the $\text{LDD}(\alpha_0, \rho_0, 0)$. Let $\text{dist}(\delta)$ be the maximal distance between the two cumulative distribution functions. Table 1 gives the values of α_δ , ρ_δ , and $\text{dist}(\delta)$ for $\alpha_0 = \rho_0 = 1$ and δ from 0 to 0.3. Of course, $\text{dist}(\delta)$ depends on α_0 , ρ_0 and

δ	0	0.03	0.06	0.09	0.12	0.15	0.18	0.21	0.24	0.27	0.30
α_δ	1	1.02	1.03	1.05	1.08	1.10	1.13	1.16	1.20	1.25	1.30
ρ_δ	1	1.01	1.02	1.04	1.05	1.07	1.09	1.12	1.15	1.19	1.24
$10^3 \text{dist}(\delta)$	0	0.64	1.35	2.12	2.98	3.94	5.02	6.24	7.63	9.24	11.11

Table 1. Parameters of LDD distributions that coincide with $\text{LDD}(1, 1, 0)$ on 0 and 1, with maximal distance between cumulative distribution functions, multiplied by 10^3 .

δ : it increases with α_0 and δ , it decreases with ρ_0 ; but its typical order of magnitude is 10^{-3} .

As a consequence, there is no hope to distinguish between LDD distributions on a sample of a few hundred data, which is the usual size in fluctuation analysis experiments. However, the observed number of mutants increases with α (the expected number of mutations), and so does the identifiability of the model. Here are the values of $\text{dist}(\delta)$ for $\delta = 0.1$, $\rho_0 = 1$, and α_0 from 10

to 50.

α_0	10	20	30	40	50
$10^3 \text{ dist}(0.1)$	14.30	21.91	27.52	32.06	35.91

The fact that more precise estimates should be obtained for large values of α rules out in our view the maximum likelihood method, as already been explained in [Hamon and Ycart \(2012\)](#); it is the main argument supporting Generating Function (GF) estimators, that allow a rescaling of the sample. The estimation method proposed below extends the GF estimators introduced in [Hamon and Ycart \(2012\)](#).

Recall the PGF of the LDD(α, ρ, δ):

$$g_{\alpha, \rho, \delta}(z) = \exp(-\alpha(1 - h_{\rho, \delta}(z))) .$$

For $0 \leq \delta < 0.5$ and $0 \leq z < 1$, we shall denote by δ_* and z_* the following quantities.

$$\delta_* = \frac{\delta}{1 - \delta} \quad \text{and} \quad z_* = \frac{z - \delta_*}{1 - z} .$$

The PGF $h_{\rho, \delta}$ and its derivatives with respect of ρ and δ , denoted by $h_{\rho, \delta}^{(\rho)}(z)$ and $h_{\rho, \delta}^{(\delta)}(z)$ respectively, are repeatedly needed in numerical procedures, so numerically stable expressions must be derived. Here are the expressions that have been implemented in our R functions.

$$h_{\rho, \delta}(z) = \delta_* + z_*(1 - \delta_*) \int_0^1 \frac{\rho v^\rho}{1 + z_* v} dv . \quad (4.13)$$

$$h_{\rho, \delta}^{(\rho)}(z) = \frac{\partial h_{\rho, \delta}(z)}{\partial \rho} = z_*(1 - \delta_*) \int_0^1 \frac{v^\rho}{1 + z_* v} (1 + \rho \log(v)) dv . \quad (4.14)$$

$$\begin{aligned} h_{\rho, \delta}^{(\delta)}(z) = \frac{\partial h_{\rho, \delta}(z)}{\partial \delta} &= \left(\frac{1}{1 - \delta} \right)^2 \left(1 - \left(z_* + \frac{1 - \delta_*}{1 - z} \right) \int_0^1 \frac{\rho v^\rho}{1 + z_* v} dv \right. \\ &\quad \left. + \frac{z_*(1 - \delta_*)}{1 - z} \int_0^1 \frac{\rho v^{\rho+1}}{(1 + z_* v)^2} dv \right) . \end{aligned} \quad (4.15)$$

We use the method of moments, such as stated by [Rémillard and Theodorescu \(2000\)](#) in a similar context. Let $0 < z_1 < z_2 < z_3 < 1$ be three different values, considered as fixed. Let g_1, g_2, g_3 be their respective images by $g_{\alpha, \rho, \delta}$. Denote by $G = G(\alpha, \rho, \delta)$ the 3-dimensional vector (g_1, g_2, g_3) . The mapping $(\alpha, \rho, \delta) \mapsto G$ is locally one-to-one, and its inverse can be used to derive

an estimate of (α, ρ, δ) from the natural estimate of G . Let $(X_n)_{n \geq 1}$ be a sequence of independent identically distributed random variables, each with PGF $g_{\alpha, \rho, \delta}$. Define the *empirical probability generating function* (EPGF) $\hat{g}_n(z)$ as:

$$\hat{g}_n(z) = \frac{1}{n} \sum_{i=1}^n z^{X_i} .$$

For $i, j = 1, 2, 3$, the expectations and covariances of $\hat{g}_n(z_i)$ and $\hat{g}_n(z_j)$ are easily expressed:

$$\mathbb{E}[\hat{g}_n(z_i)] = g_{\alpha, \rho, \delta}(z_i) ,$$

and

$$\text{cov}[\hat{g}_n(z_i), \hat{g}_n(z_j)] = c(z_i, z_j) = g_{\alpha, \rho, \delta}(z_i z_j) - g_{\alpha, \rho, \delta}(z_i) g_{\alpha, \rho, \delta}(z_j) .$$

Consider the 3-dimensional vector

$$\hat{G} = (\hat{g}_n(z_1), \hat{g}_n(z_2), \hat{g}_n(z_3)) .$$

Its coordinates are empirical means of independent, identically distributed, bounded random variables: hence it is a strongly consistent estimator of G . By the Central Limit Theorem, $\sqrt{n}(\hat{G} - G)$ converges in distribution to the trivariate centered normal distribution, with covariance matrix $C = (c(z_i, z_j))_{i,j=1,2,3}$. [Rémillard and Theodorescu \(2000, Proposition 3.1\)](#) give a stronger result, stating the functional convergence of $\hat{g}_n(z)$ to a Gaussian process.

The Jacobian matrix of G as a function of (α, ρ, δ) is the following.

$$\begin{aligned} J &= \begin{pmatrix} \frac{\partial g_{\alpha, \rho, \delta}(z_1)}{\partial \alpha} & \frac{\partial g_{\alpha, \rho, \delta}(z_1)}{\partial \rho} & \frac{\partial g_{\alpha, \rho, \delta}(z_1)}{\partial \delta} \\ \frac{\partial g_{\alpha, \rho, \delta}(z_2)}{\partial \alpha} & \frac{\partial g_{\alpha, \rho, \delta}(z_2)}{\partial \rho} & \frac{\partial g_{\alpha, \rho, \delta}(z_2)}{\partial \delta} \\ \frac{\partial g_{\alpha, \rho, \delta}(z_3)}{\partial \alpha} & \frac{\partial g_{\alpha, \rho, \delta}(z_3)}{\partial \rho} & \frac{\partial g_{\alpha, \rho, \delta}(z_3)}{\partial \delta} \end{pmatrix} \\ &= \begin{pmatrix} g_1(h_{\rho, \delta}(z_1) - 1) & g_1 \alpha h_{\rho, \delta}^{(\rho)}(z_1) & g_1 \alpha h_{\rho, \delta}^{(\delta)}(z_1) \\ g_2(h_{\rho, \delta}(z_2) - 1) & g_2 \alpha h_{\rho, \delta}^{(\rho)}(z_2) & g_2 \alpha h_{\rho, \delta}^{(\delta)}(z_2) \\ g_3(h_{\rho, \delta}(z_3) - 1) & g_3 \alpha h_{\rho, \delta}^{(\rho)}(z_3) & g_3 \alpha h_{\rho, \delta}^{(\delta)}(z_3) \end{pmatrix} \end{aligned}$$

Admitting that J is non singular, denote by ϕ the inverse of the mapping $(\alpha, \rho, \delta) \mapsto G$.

Then $\phi(\hat{G})$ is a consistent estimator of (α, ρ, δ) . By Slutsky's theorem, such as formulated by

(Rémillard and Theodorescu, 2000, Theorem 3.4), $\sqrt{n}(\phi(\hat{G}) - (\alpha, \rho, \delta))$ converges in distribution to the trivariate centered normal distribution with covariance matrix $(J^{-1})^t C J^{-1}$. From there, confidence intervals and p-values of hypothesis testing can be obtained by standard procedures (see e.g. Anderson (2003)). As was explained in Hamon and Ycart (2012), the main advantage of GF estimators is to allow a rescaling of the sample, which makes the method applicable to large values of α . The idea is to replace z by $z^{1/b}$ in the definition of $\hat{g}_n(z)$:

$$\hat{g}_n(z^{1/b}) = \frac{1}{n} \sum_{i=1}^n (z^{1/b})^{X_i} = \frac{1}{n} \sum_{i=1}^n z^{X_i/b}.$$

We propose to choose for b the q^{th} quantile of the sample, where q was experimentally set to 0.1.

The estimator $\phi(\hat{G})$ is theoretically consistent. However, for intrinsic reasons that were explained at the beginning of this section, it is numerically unstable, and can only be applied to very large samples, beyond the size of those usually collected in fluctuation analysis experiments. Thus we have been led to propose other estimators, that will now be described.

We first assume that δ is known. Observe that for $z = \delta_*$, $z_* = 0$ and $h_{\rho, \delta}(\delta_*) = \delta_*$: $h_{\rho, \delta}$ has a fixed point at δ_* , independently of ρ . Therefore $\hat{g}_n(\delta_*)$ converges to $g_{\alpha, \rho, \delta}(\delta_*) = \exp(\alpha(\delta_* - 1))$. Hence $\log(\hat{g}(\delta_*))/(\delta_* - 1)$ is a consistent estimator of α , that we shall call the *fixed point estimator*. It does not depend on ρ . For $\delta = 0$, the fixed point estimator is $-\log(\hat{g}(0))$, and $\hat{g}(0)$ is the proportion of zeros in the sample. Thus the fixed point estimator extends the so called p_0 -method, initially proposed by Luria and Delbrück (1943) (see Foster (2006) for a review on estimation methods for the LD(α, ρ)). The p_0 -method, even though it gives acceptable results for low values of α , cannot be applied for large α 's: the same can be said of the fixed point estimator.

The best results were obtained for the GF estimators that were developed in Hamon and Ycart (2012) for the LD(α, ρ) case. We briefly recall their definition below.

Consider the following ratio.

$$f_{z_1, z_2}(\rho) = \frac{h_{\rho, \delta}(z_1) - 1}{h_{\rho, \delta}(z_2) - 1}.$$

The function that maps ρ onto $y = f_{z_1, z_2}(\rho)$ is continuous and strictly monotone, hence one-to-one. Therefore the inverse, that maps y onto $\rho = f_{z_1, z_2}^{-1}(y)$, is well defined. For $0 < z_1 < z_2 < 1$, let $\hat{y}_n(z_1, z_2)$ denote the following log-ratio.

$$\hat{y}_n(z_1, z_2) = \frac{\log(\hat{g}_n(z_1))}{\log(\hat{g}_n(z_2))}.$$

An estimator of ρ is obtained by:

$$\hat{\rho}_n(\delta) = f_{z_1, z_2}^{-1}(\hat{y}_n),$$

then an estimator of α by:

$$\hat{\alpha}_n(\delta) = \frac{\log(\hat{g}_n(z_3))}{h_{\hat{\rho}_n(z_1, z_2)}(z_3) - 1}.$$

The asymptotic covariance matrix of $(\hat{\alpha}_n, \hat{\rho}_n)$ given in Proposition 4.1 of [Hamon and Ycart \(2012\)](#) is still valid here, replacing h_ρ and its derivative in ρ by $h_{\rho, \delta}$ and $h_{\rho, \delta}^{(\rho)}$. Scaling by a quantile of the sample as was explained above can also be applied.

If we assume now that ρ is known and δ unknown, the estimators described above are easily adapted, by exchanging the roles of ρ and δ , and replacing $h_{\rho, \delta}^{(\rho)}$ by $h_{\rho, \delta}^{(\delta)}$. New GF estimators $\hat{\alpha}_n(\rho)$ and $\hat{\delta}_n(\rho)$ are obtained.

In practice, neither δ nor ρ can be supposed to be known. For a given value of δ , consider the estimators $\hat{\alpha}(\delta)$ and $\hat{\rho}(\delta)$ described above. The distributions $\text{LDD}(\hat{\alpha}(\delta), \hat{\rho}(\delta), \delta)$ from different values of δ are not far from each other. To distinguish between them, we propose to use as an estimator of δ the value $\hat{\delta}$ that minimizes the distance between the theoretical PGF and the EPGF of the sample (up to possible rescaling). We shall denote by $\hat{B} = (\hat{\alpha}(\hat{\delta}), \hat{\rho}(\hat{\delta}), \hat{\delta})$ this new estimator. Unlike $\phi(\hat{G})$ (which is numerically unstable for small samples), \hat{B} can be calculated on samples of any size. It will be shown in the next section that when both can be calculated, \hat{B} has a better Mean Squared Error (MSE) than $\phi(\hat{G})$.

5. EXPERIMENTAL RESULTS

Using extensively the simulation procedure described in section 3, we have conducted different simulation experiments in order to assess the qualities of the estimators proposed in section 4. We have also used the most comprehensive set of data available so far (1102 values), that of *Boe and others* (1994). Our main conclusions are reported in this section.

The reason why the GF estimator $\phi(\hat{G})$ cannot be calculated for small samples was explained in the previous section. The question arises to compare it, on large samples and for large values of α , to the GF estimator \hat{B} obtained by estimating first α and ρ on different values of δ , and then selecting the set of parameters that minimizes the distance between PGF's. The second one consistently gives better results. Here are for instance the MSEs on the estimation of the three parameters, on 1000 simulated samples of size 10^4 of the LDD(10, 1, 0.1).

	α	ρ	δ
$\phi(\hat{G})$	0.551	0.019	0.111
\hat{B}	0.481	0.016	0.079

Notice that both estimators perform quite poorly on the estimation of δ : the MSE is comparable to the true value. As explained in the previous section, this must be blamed on the intrinsic lack of identifiability of the model, rather than the estimators.

We then tried to evaluate the quality of different estimators of α , which is the parameter of interest in fluctuation analysis. Four estimators were tried on 1000 samples of size 1000 of the LDD(α, ρ, δ), computing for each estimator the MSE on α .

- GFd: the estimate $\hat{\alpha}(\delta)$ obtained using the true value of δ ;
- GFr: the estimate $\hat{\alpha}(\rho)$ obtained using the true value of ρ ;
- GF0: the first coordinate $\hat{\alpha}$ of \hat{B} (no prior information);
- FP: the fixed point estimator $\frac{\log(\hat{g}(\delta_*))}{\delta_* - 1}$ (using the true value of δ).

(α, ρ, δ)	GFd	GFr	GF0	FP
(1, 1, 0.1)	0.040	0.087	0.136	0.041
(1, 1, 0.05)	0.039	0.076	0.144	0.041
(1, 0.8, 0.05)	0.042	0.105	0.166	0.044
(10, 1, 0.1)	0.272	0.506	1.000	2.677
(10, 1, 0.05)	0.894	1.143	1.983	11.84
(10, 0.8, 0.05)	1.000	1.393	2.088	13.87

Table 2. Mean Squared Errors on 4 estimators of α from 1000 samples of size 1000 of the LDD(α, ρ, δ) for different values of the parameters. The first estimate uses the true value of δ , the second one the true value of ρ , the third one uses no prior information. The last one (fixed point) does not depend on ρ and uses the true value of δ .

Table 2 shows the MSEs obtained for different sets of parameters. Not surprisingly, using the true value of δ or ρ gives a better estimate of α ; the information on δ yields a better precision than the information on ρ . Both estimators GFd and FP use the information on δ , but the first one is better. For low values of α , FP performs reasonably well, as does the p_0 -method for $\delta = 0$. However for large values of α , FP is strongly biased. A value of ρ smaller than 1 implies a heavier tail, hence larger and more frequent outliers. It worsens the estimation of α , whatever the estimator.

Apart from simulation experiments, we have tried estimating α, ρ, δ on several samples of real data. The results obtained on the 1102 data from [Boe and others \(1994\)](#) are reported here. These data were adjusted on the LD(α, ρ) by [Zheng \(2005\)](#) using the maximum likelihood method: his estimates of α and ρ are 0.71 and 0.84 respectively. Table 3 shows the estimates of α and ρ obtained for values of δ ranging from 0 to 0.3. It also gives the distances between the empirical distribution and the estimated LDD, either in the sense of PGF's or in that of cumulative distribution functions. The fit is quite good, whatever the value of δ . The value $\delta = 0.06$ gives the best fit in the sense of PGF's. Even though this value is coherent with death probability estimates reported by [Fontaine and others \(2008\)](#), it cannot be considered as reliable. Indeed, the 95% confidence margin of error on δ , deduced from the asymptotic covariance matrix, is ± 0.13 . This huge margin is coherent with what we have observed on simulated samples with analogous

parameters.

δ	0.00	0.03	0.06	0.09	0.12	0.15	0.18	0.2	10.24	0.27	0.30
$\hat{\alpha}(\delta)$	0.71	0.72	0.73	0.75	0.77	0.79	0.81	0.83	0.87	0.90	0.95
$\hat{\rho}(\delta)$	0.82	0.83	0.84	0.84	0.85	0.86	0.87	0.88	0.90	0.92	0.94
DG	1.04	0.87	0.74	0.79	0.86	0.94	1.05	1.19	1.38	1.66	2.06
DF	6.48	6.25	6.29	6.53	6.79	7.08	7.41	7.79	8.23	8.74	9.35

Table 3. Estimates of α and ρ for different values of δ on the set of data of [Boe and others \(1994\)](#). On row 4, DG is the maximal distance between the EPGF function of the sample and the PGF of the LDD($\hat{\alpha}(\delta), \hat{\rho}(\delta), \delta$), multiplied by 10^3 . On row 5, DF is the distance between cumulative distribution functions, also multiplied by 10^3 .

The main conclusion of our experimental study is that the death probability δ cannot be reliably estimated on samples such that the product $\alpha \times n$ is lower than 10^5 , which is far beyond current fluctuation analysis experiments. We remark that available estimates of δ have orders of magnitude of a few percents: see [Fontaine and others \(2008\)](#). Table 1 for theoretical distances as well as Table 3 for actual data, permit to evaluate the influence of δ : it turns out that the effect of a small δ on estimates on the estimates of α and ρ has the same order of magnitude as δ itself. So neglecting the effect of cell deaths if no reliable estimate of their probability is available, seems legitimate.

6. CONCLUSION

A probabilistic model of fluctuation analysis, taking into account cell deaths, has been proposed. A new family of distributions LDD(α, ρ, δ), modeling asymptotic number of mutants has been derived. The three parameters are the expected number of mutations α (which is the parameter of interest in fluctuation analysis), the relative fitness of normal cells compared to mutants ρ , and the death probability of mutant cells δ . In the particular case $\delta = 0$, the classic Luria-Delbrück distribution is recovered. The extension of known mathematical results to the case $\delta > 0$ is straightforward: explicit simulation and computation algorithms for probabilities have been described. The LDD(α, ρ, δ) has the same type of asymptotic behavior than the Luria-

Delbrück distributions: heavy tail with tail exponent ρ . Thus, the occurrence of “jackpots” (large counts of mutants) is a common feature. Modeling an observed sample of mutant counts by a $\text{LDD}(\alpha, \rho, \delta)$ poses the problem of estimating the three parameters simultaneously. If the death probability δ is known, then α and ρ can be estimated using the generating function method, exactly as in the case $\delta = 0$. The larger the expected number of mutations α , the more precise the estimates. However, for samples of size smaller than 10^3 , all values of δ lead to a good fit, and the different distributions so obtained can hardly be distinguished. Choosing for δ the value giving the best fit yields a consistent estimator with optimal mean squared error, but it cannot be considered a reliable choice for small samples. Since the death probabilities that have been reported in practice are small, their influence on the estimates of α and ρ can be neglected as a first approximation, if no prior information on the actual value of δ is available. A script containing the R functions for the statistical treatment of the LDD distributions has been made available online.

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